PCR amplification of DNA methylated with the DamID technique
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Please check http://www.nki.nl/nkidep/vansteensel for updated versions of this protocol.

DpnI digestion of plasmid competitor DNA

This is the plasmid encoding the Dam fusion protein that was used to transfect the cells. Per hybridization 25 ug of competitor plasmid (measured on a spectrophotometer) is needed.
Note: DamID with lentiviruses or stable lines does not require addition of competitor plasmid to the hybridization.

DpnI lot no: 10x buffer 4 lot no:

…	ul	(27 ug) plasmid DNA
10	ul	10x buffer 4 (NEB)
3	ul	DpnI (NEB, 20 U/ul)
…	ul	ddH_2O
100	ul	

- Incubate over night at 37 °C
- Clean up the digested plasmid: use Qiagen PCR purification columns (according to manufacturers protocol) or Phenol/ Chloroform extract and ethanol precipitate the digests.

Precipitation of gDNA

Measure the concentration and the volume of the gDNA on the Nanodrop.

DNA sample – concentration – volume - total ug

Precipitate all the gDNA:
- Add … ul (2 volumes) 100% EtOH,
- Add … ul (0.1 volume) sodium acetate (3M) pH 5.5 and mix.

- Put 30 mins at -20 °C
- Spin for 30 mins at max rpm and at 4 °C
- Remove the sup and wash the pellets with … ul (1 volume) 70% EtOH.
- Spin again 5 mins at max rpm and at 4 °C
- Remove sup and air dry the pellets.
- Dissolve the pellets in $T_{10}E_{0.1}$ (pH7.5) to 1 ug/ul.
- Heat to 55 °C for 30 mins, vortex after 15 mins.
- Make sure the gDNA is dissolved.

Confirm the concentration of the samples:

- Measure 1 ul on the Nanodrop.

DpnI digestion of gDNA

DpnI digest 2.5 ul gDNA (=2.5 ug); include a control digestion without DpnI enzyme and a control digestion (with DpnI) that will be used as a ligase negative control.

- Add 7 ul (6 ul ddH_2O + 1 ul buffer 4) mix per tube.
- Add 0.5 ul DpnI enzyme to each digestion (add 0.5 ul ddH_2O to the control(s) without DpnI enzyme)

DpnI lot no: 10x buffer 4 lot no:

2.5	ul	gDNA (2.5 ug)
1	ul	buffer 4 (NEB, 10x)
0/0.5	ul	10 units DpnI (NEB, 20 U/ul)
6.5/6	ul	ddH_2O
10	ul	

- Digest over night, 37 °C. (4h digestion is also possible).
- Inactivate DpnI for 20 min at 80 °C

Ligation of adaptors

The DpnI digested gDNA is ligated with the adaptor AdR (sequence see general notes).

- Make a stock of ds AdR adaptor by pipetting 50 ul of AdRt (100 uM) and 50 ul of AdRb (100 uM) together. Vortex and place it into a beaker with water that just has boiled. Let the beaker cool to room temperature, so the adaptors can anneal slowly.

- Make a mix (0.8 ul AdR, 2 ul ligase buffer and 6.2 ul ddH_2O per sample) and add 9 ul to each tube.
- Add 1 ul T4 ligase per sample (add 1 ul ddH_2O to the ligase-negative control.)

T4 ligase lot no: 10x lig. buffer lot no:

10	ul	DpnI digested gDNA (2.5 ug)
2	ul	10x ligation buffer (Roche)
0.8	ul	ds adaptor AdR (50 uM)
0/1	ul	T_4 ligase (5U/ul, Roche)
7.2/6.2	ul	ddH_2O
20	ul	

- Ligate for 2 h at 16°C (ligation over night is also possible).
- Inactivate T4 ligase for 10 minutes at 65°C.

DpnII digestion

This digestion is to destroy fragments that have unmethylated GATCs.

- Make a mix of DpnII buffer and ddH_2O and add 29 ul per sample.
- Add 1 ul DpnII per sample.

DpnII lot no: 10x DpnII buffer lot no.:

20	ul	"ligation"
5	ul	10x DpnII buffer (NEB)
1	ul	DpnII (NEB, 10 U/ul)
24	ul	ddH_2O
50	ul	

- Digest 1h at 37°C.

PCR

!!Beware of the risk of contamination, especially when handling PCR products!!

Include a PCR negative control reaction. Make a master-mix including the PCR enzyme.

PCR advantage enzyme mix lot no: 10x cDNA PCR reaction buffer lot no:

10	ul	DpnII digested DNA (0.5 ug)
5	ul	10x cDNA PCR reaction buffer (Clontech)
1.25	ul	primer Adr-PCR (50 uM)
4	ul	dNTPs (2.5 mM each)
1	ul	PCR advantage enzyme mix (Clontech, 50x)

28.75	ul	ddH_2O
50	ul	

PCR cycling (program DAMPCR)

step	°C	min	
1)	68	10	
2)	94	1	
3)	65	5	
4)	68	15	
5)	94	1	
6)	65	1	
7)	68	10	
8)	goto step 5		3x
9)	94	1	
10)	65	1	
11)	68	2	
12)	goto step 9		14x
13)	4	for ever	
14)	END		

Note: For DamID with lentiviruses the number of PCR cycles should be increased. Add 3 extra cycles to step 12 (goto step 9, **17**x instead of 14x).

DpnII digest 8 ul of the PCR products.

8	ul	PCR product
2	ul	10x DpnII buffer (NEB)
0.8	ul	DpnII (NEB, 10 U/ul)
9.2	ul	ddH_2O
20	ul	

- Digest 1h at 37°C.
- Analyze on a 1% agarose gel.

Clean up DNA

Clean the PCR reactions of the samples that are going to be hybridized with the Qiaquick PCR purification kit according to the manufacturers protocol.

General notes

Concentration measurements are done on the Nanodrop spectrophotometer. The DNA amounts used in this protocol are based on Nanodrop measurements.
Alternatively, concentration measurements can be done with a Fluorimeter (DyNA 200 from Hoefer) with Hoechst using herring sperm DNA for standard calibration. Use twice the amount of DNA indicated in the protocol when the Fluorimeter is used. For example: Dpn1 digest 5 ug (Hoechst) in stead of 2.5 ug gDNA (nanodrop).

Right adaptor, top strand, NOT 5' phosphorylated
AdRt 5' CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGA 3'

Right adaptor, bottom strand, NOT 5' phosphorylated
AdRb 5' TCCTCGGCCG 3'

PCR primer for right adaptor for library cloning (Tm=67.7)
AdR_PCR 5' GGTCGCGGCCGAGGATC 3'

Ordering information

PCR amplification of DNA methylated with the DamID technique:

NEB	DpnI + buffer 4	20 000 U/ml	R0176L
NEB	DpnII + DpnII buffer	10 000 U/ml	R0533S
Roche	T_4 Ligase + 10x buffer	500 U	799099
BD biosciences	Adv. cDNA polymerase mix	100 rxns	8417-1